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Direct UV-MALDI-TOF MS Analysis of (Glyco)proteins of Fractions of Bovine Seminal Plasma

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Bovine seminal plasma was submitted to chromatography on Con A-Sepharose. The "non-interacting", "weakly-interacting" and "strongly-interacting" fractions were analyzed through UV-MALDI-TOF MS together with a subfraction of the "non-interacting" material, using sinapinic acid (SA) as matrix. The spectra were obtained in linear positive mode in the 4.0–90.0 kDa mass/charge range showing peaks in well defined zones, namely: 5.5–8.0 kDa, 10.0–12.0 kDa, 12.5–14.0 kDa (major), 23.2–23.7 kDa, 26.1–27.5 kDa and 38.0–40.0 kDa. High sensitivity spectra showed some very small peaks until 90 kDa. Bovine seminal protein (BSP-A3), acidic seminal fluid protein (αSFP) and PDC-109 glycoproteins (BSP-A1 and BSP-A2) were identified. Caltrin, the human epididymis-specific glycoprotein (HE4), the calcium transport inhibitor protein, the inhibitor of metalloprotease 2 (TIMP-2), osteopontin (OPN) and the prostatic acid protease (PAP) were tentatively identified. The molecular weight of some peaks can be arranged in a sequence from that of BSP-A3 going through the molecular weights of glycoforms (including the known BSP-A1 and BSP-A2) which differ in the amounts of neutral hexoses and sialic acids, composing a BSP-family more extended than previously reported. Another two families could be builded up from proteins of molecular weight of about 12730 and 12750 Da and glycoforms which differ from them also by hexoses and sialic acids. The structures of the deduced O-linked oligosaccharides of the glycoforms are in complete agreement to that determined for the BSP-A1

Abbreviations : UV-MALDI-TOF MS, ultraviolet matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; m.w., molecular weight; (BSP), bovine seminal plasma; (BSP-A3), bovine seminal protein; (PDC-109), complex formed by glycoproteins BSP-A1 and BSP-A2; (aSFP), acidic seminal fluid protein; (TIMP-2), inhibitor of metalloprotease 2; (PSA), prostate specific antigen; (PAP), prostatic acid phosphatase; (FAA), fertility-associate antigen; (CaTIP), calcium transport inhibiter protein; (HE4), human epididymis-specific glycoprotein; (OPN), osteopontin.

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oligosaccharide. Small differences in the m. w. of some (glyco)proteins were attributed to genetic polymorphysm. The identification of proteins and O-linked glycoproteins in the "interacting" fractions of the chromatography suggests that the fractionation was not due to specific affinity interactions but to non-specific hydrophobic interactions of the proteins with the hydrophobic pocked of the Con A.

Keywords : bovine seminal plasma, nor-harmane, sinapinic acid, (glyco)proteins, UV-MALDI-TOF MS

INTRODUCTION

Mammalian seminal plasmas, the combined secretions of the male accessory organs of reproduction are extremely, chemically and physicochemically, complex fluids containing a wide variety of (glyco)proteins of different molecular masses. This (glyco)protein composition varies from specie to specie but the fluid has always important effects on the sperm function (Cross, 1993; Calvete et al., 1996). Mammalian fertility-associated (glyco)proteins participate in sperm-egg recognition, modulation of capacitation of the spermatozoon, the acrosome reaction, etc. (Calvete et al., 1996; Killian et al., 1993; Martin Ruiz et al., 1998; Yonezawa et al., 1997). Some of the bovine seminal plasma (glyco)proteins are considered markers associated with bull fertility (Killian et al., 1993).

Seminal plasmas have been fractionated using different methodologies (Calvete et al., 1996) and its compositions, or that of its fractions, were studied mainly by D or 2D SDS-PAGE techniques (Starita-Geribaldi et al., 2001), nevertheless relatively few (glyco)proteins of these origin have been isolated and characterized (Calvete et al., 1996; Kameswari and Prasad, 1994; Lewis et al., 1985; Sitaram et al., 1986; Manjunath and Sairam, 1987).

The usefulness of UV-MALDI MS for the study of proteins and glycoproteins is well known (Cristoni and Rossi Bernardi, 2003; Zaia, 2004). Pure samples of major (glyco)proteins from bovine seminal plasma have been analyzed by UV-MALDI MS showing molecular weights in the range, but always somewhat lower, than those minimal determined through SDS-PAGE (Calvete et al., 1996). Samples extracted from bovine epididymis and ejaculated spermatozoa were analyzed by UV-MALDI MS and the major peaks, without further characterization, were correlated with different spermatic parameters (Silva et al., 2003). UV-MALDI MS analysis of the protein triptic digests allowed the identification of some major proteins and peptides of the human seminal plasma (Overberg et al., 1990; Fung et al., 2004).

In the present study the bovine seminal plasma has been fractionated by chromatography through Con A - Sepharose. Three fractions were obtained, namely: a) the "non-interacting" material that would contain, according to the specificity of the lectin, proteins together with O-linked oligosaccharides and some tri and tetraantenary complex type N-linked oligosaccharides-containing glycoproteins, b) that eluted with methyl α-D-glucopyranoside which would contain N-linked biantennary complexes binding weakly to Con A, and c) the high mannose and hybrid type N-linked chains-containing glycoproteins which would strongly bind to the column and are eluted only with methyl α-D-mannopyranoside (Marquinez et al., 2000). With the interest of producing fractions having inhibitory effects on the acrosine-like protease activity, the non-interacting fraction was further subfractionated permitting the characterization of the bovine acidic seminal fluid protein (αSFP) and the glycosylated forms of the bovine seminal protein (PDC-109) (Marquinez et al., 2000).

We report now, the UV-MALDI-TOF MS study of the three fractions from the Con A-Sepharose chromatography and of a selected subfraction of the "non-interacting" material (fraction II) (Fig. 1). In this last case (fraction II) the MALDI results were compared with those obtained previously (Marquinez et al., 2000) and in this work, through SDS-PAGE, "in gel" triptic digest

and microsequencing.

MATERIALS AND METHODS

Starting material

Semen samples, obtained from Holando-Argentina bulls with an artificial vagina, were pooled and, after liquification, centrifuged (6,000 g, 15 min., 5ºC) to eliminate the spermatozoa. The supernatant (seminal plasma) was recovered and centrifuged at 10,000 g for 60 min at 5ºC, dialyzed with dialysis bags of cutt off 12000 Da, and freeze-dried.

Fractionation process

A Concanavalin A (Con A)-Sepharose column was prepared as previously reported for bovine seminal plasma (Marquinez et al., 2000) and for human seminal plasma (Marquinez et al., 2003; Lelli and Scacciati de Cerezo, 1999). The bovine seminal plasma (200 mg) was dissolved in phosphate buffer saline (PBS) (50 mM phosphate buffer pH 7, 200 mM NaCl) and applied at the top of the column. This was eluted with PBS until absence of proteins (Lowry et al., 1957) and carbohydrates (Dubois et al., 1956) (fraction 1). The interacting material was eluted first with PBS containing 5 mM methyl α-D-glucopyranoside (fraction 2) and then with PBS containing 200 mM methyl α-D-mannopyranoside (fraction 3). In the fractions eluted with the inhibitory sugars only proteins were determined. The material eluted with each buffer was combined, dialyzed with dialysis bags of cut off 12000 Da, freeze-dried and kept at −20ºC. The Con A non-interacting fraction (fraction 1) was chromatographed through a Sephadex- G-50 column. The highest m.w. Sephadex-fraction (62 kDa) was chromatographed in a *Phaseolus vulgaris* leucoagglutinin (L-PHA)-agarose affinity column. The non-interacting material, (fraction II) (Fig. 1) was employed as starting material for FPLC chromatographic column (Fig. 7). Peaks IIb and IIc of the FPLC column were analyzed by SDS-PAGE, "in gel" digestion and sequenciation, as well as the small shoulder (IIa') of the major (void volume) peak IIa (Fig. 7). The "non- interacting" (1), "weakly interacting" (2) and "strongly interacting" (3) fractions of bovine seminal plasma obtained through chromatography on Con A-Sepharose and fraction II (Fig. 1), obtained by gel-permeation on Sephadex G-50 and further affinity chromatography on *Phaseolus vulgaris*-agarose from the "non-interacting" fraction, were used for the UV-MALDI-TOF MS analysis (Fig. 1).

UV-MALDI-TOF MS analysis

Matrices for UV-MALDI-TOF MS: The β-carbolines (9H-pyrido[3,4-*b*]indole) *nor*-harmane (nor-Ho) and (1-methyl-7-methoxy-3,4-dihydro-9H-pyrido[3,4-*b*]indole) harmaline (Ha) (Nonami et al., 1997; Nonami et al., 1998; Erra-Balsells and Nonami, 2002), 2,5-dihydroxybenzoic acid (DHB, gentisic acid, GA), 2,4,6-trihydroxyacetophenone (THAP), trans 3,4-dimethoxy-4-hydroxycinnamic acid (sinapinic acid, SA), trans α-ciano-4-hydroxycinnamic acid (CHC), 5-chloro-2-mercaptobenzothiazole (CMBT) were obtained from Aldrich Chemical Co. *Calibrating chemicals for UV-MALDI-TOF MS analysis*: bovine insulin (mw 5733.5), myoglobin (mw 16950 and double charged cation at m/z 5965) and bovine albumin (mw 66525 and double charged cation at m/z 33262.5) were purchased from Sigma-Aldrich. Spectra were calibrated using as external calibration reagents the above commercial proteins with *nor*-harmane as matrix in positive- and in negative-ion mode. The Kratos Kompact and the Bruker Flexcontrol 2.4 software package programs for calibration were used.

Solvents: Methanol and acetonitrile (Sigma-Aldrich HPLC grade), trifluoroacetic acid (TFA, Merck) were used as purchased without further purification. Water of very low conductivity (Milli Q grade; 56–59 nS/cm with PURIC-S, ORUGANO Co., Ltd., Tokyo, Japan) was used.

UV-MALDI-TOF MS experiments: Measurements were performed using: (i) a Shimadzu Kratos, Kompact MALDI 4 (pulsed extraction) laser-desorption time-of-flight mass spectrometer (Shimadzu, Kyoto, Japan) equipped with a pulsed nitrogen laser (λ_{em} = 337 nm; pulse width = 3 ns),

Table 1 UV-MALDI-TOF-MS analysis of bovine seminal plasma with different matrices and using mixture and sandwich sample preparation methods.

	Matrix						
Sample preparation method	SA	Ha	GA	CHC	Nor-Ho	THAP	CMBT
Sandwich (Method A)	+/−	+/−	+	+	+/(−)	(+)	0
Mixture (Method B; 1:4 v/v)	(+/−)	0	0	0	0	0	0
Mixture (Method B; 1:8 v/v)	0	0	0	0	0	0	0

No spectrum signal 0; spectrum obtainable in positive mode +; in negative mode −; both modes +/−. Signals in between parenthesis were low intensity signals.
Matrices: SA = Sinapinic acid, Ha = Harmaline, GA = Gentisic acid, CHC = α-cyano-4-hydroxycinnamic acid, THAP = 2,4,6-trihydroxyacetophenone, nor-Ho = nor-Harmane, CMBT = 5-chloro-2-mercaptobenzothiazole.
Sample preparations and solutions concentration: see Materials and Methods.

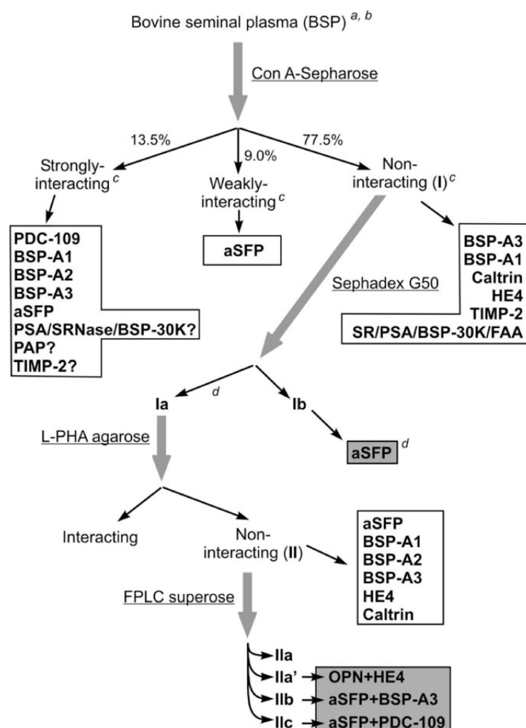

Fig. 1 Fractionation of the bovine seminal plasma: (glyco)proteins in white boxes were characterized by UV-MALDI-MS, while those in grey boxes were identified in this or previous paper (Marquinez et al., 2000) through SDS-PAGE, tripsin digest and microsequencing. [a] Obtained from a pool of semen samples. [b] After liquefaction (natural proteolysis) and dialysis with 12 kDa cut-off tubing. [c] After a new dialysis in the same conditions as above. [d] From ref (Marquinez et al., 2000).

tunable PDE, PSD (MS/MS device) and a secondary electron multiplier (SEM) and (ii) a Bruker Daltonics OmniFLEX UV-MALDI-TOF Mass Spectrometer (Bruker, USA) equipped with a pulsed nitrogen laser (λ_{em} = 337 nm), with tunable pulsed-delayed extraction time (PDE).

Experiments were performed using firstly the full range setting for laser firing position in order to select the optimal position for data collection, and secondly fixing the laser firing position in the sample sweet spots. The samples were irradiated just above the threshold laser power for obtaining molecular ions and with higher laser power for studying matrix cluster formation. Thus, the irradiation used for producing a mass spectrum was analyte-dependent with an acceleration voltage of 20 kV. Usually 50–100 spectra were accumulated. All samples were measured in the linear and

the reflectron modes, in both positive- and negative-ion mode. The spectra obtained in the linear, positive ion-mode were used and only the peaks clearly distinguishable from the matrix baseline were taken into account. The stainless steel polished surface twenty-sample-slides were purchased from Shimadzu Co., Japan (P/N 670-19109-01). Polished surface slides were used in order to get better images for morphological analysis with a stereoscopic microscope (NIKON Optiphot, Tokyo, Japan; magnification×400) and with a high-resolution digital microscope (Keyence VH-6300, Osaka, Japan; magnification×800).

Sample preparation: Matrix stock solutions were made by dissolving 10 mg of the selected compound in 1 ml of 2:3 (v/v) MeCN/0.1% TFA in water. Analyte solutions were freshly prepared by dissolving the samples (0.15 mg) in 0.1% TFA in water (1 ml).

To prepare the analyte-matrix deposits two methods were used (Nachbar et al., 1976; Shaltiel, 1977; Yamamoto et al., 1993). *Method A*: (*sandwich method*), typically 0.5 μl of the matrix solution (M) was placed on the sample probe tip, and the solvent removed by blowing air at room temperature. Subsequently, 0.5 μl of the analyte solution (A) was placed on the same probe tip covering the matrix and partially dissolving it, and the solvent was removed by blowing air. Then, two additional portions (0.5×2 μl) of the matrix solution (M) were deposited on the same sample probe tip, producing a partial dissolution of the previously deposited thin-film matrix and analyte layers. The matrix to analyte ratio was 4:1 (v/v) and the matrix and analyte solution loading sequence was: i) matrix, ii) analyte, iii) matrix, iv) matrix (M: A: M: M). Sandwich method was also used combining different matrices as follows: GA: A: SA: SA, SA: A: GA: GA and CMBT: A: GA: GA (see Table 2). *Method B* (*mixture method*), the analyte stock solution was mixed with the matrix solution in 1:1 to 1:12 v/v ratio. A 0.5 μl aliquot of this analyte-matrix solution was deposited onto the stainless steel probe tip and dried with a stream of forced room temperature air. Then, an additional portion of 0.5 μl was applied to the dried solid layer on the probe, causing it to re-dissolve partially, and the solvent was removed by blowing air.

Chromatographic studies: Fast Protein Liquid Chromatography (FPLC).

A 100 μl of Tris-HCl buffer pH 8 solution (5 mg/ml) of the flow-through material of the L-PHA agarose column (Fig. 1) was injected in a Superose 12 HR Fast Protein Liquid Chromatography (FPLC) (Amersham Pharmacia, Uppsala, Sweden). Elution was performed with Tris-HCl buffer, pH 8. The flow rate was 0.5 ml/min.

The column was calibrated with a solution of 3 mg/ml of each, Blue Dextran (Mr 2000000), amylase (Mr 200000), ovoalbumin (Mr 45000) and carbonic anhidrase (Mr 29000) as molecular mass standards. The four fractions obtained IIa, IIa′ IIb and IIc were separately analyzed (Fig. 7).

Sodium dodecylsulfate-polyacrilamide gel electrophoresis (SDS-PAGE):

Denaturing and reducing conditions (Laemmli, 1970) were used with a discontinuous gel as described in a previous work (Marquinez et al., 2000). Resolving gel was 12.5%T, 2%C in 1 mM Tris buffer (1 mM Tris with 0.9% glycine and 1% SDS). Running conditions were 100 V in the stacking and 200 V in the resolving gels. T=(g acrilamide+gBis)/100 ml solution; C=g Bis% T. Gels were stained with Coommassie Brilliant Blue or Schiff reagents. For sequence determination studies the SDS-PAGE was performed in a Mini Protean II system from Bio Rad as described pre-

Table 2 UV-MALDI-TOF-MS analysis of bovine seminal plasma combining different matrices and using sandwich sample preparation method.

Sandwich method (M: A: M: M)		
GA: A: SA: SA	SA: A: GA: GA	CMBT: A: GA: GA
+/−	0	0

No spectrum signals 0; spectrum obtainable in positive mode+; in negative mode −; both modes+/−.
Matrices (M): SA=Sinapinic acid, GA=Gentisic acid, CMBT=5-chloro-2-mercaptobenzothiazole; Analite (A). Sample preparation and solutions concentration: see Materials and Methods.

viously (Marquinez et al., 2000). The gel employed and the running conditions were similar to those described before.

"In gel" digestion

After SDS-PAGE the "in gel" digestion was performed according to Rosenfeld (Rosenfeld et al., 1992). The band to be digested was cut, introduced in an Eppendorf tube, washed twice with 150 μl of a mixture of acetonitrile 200 mM- ammonium carbonate (1:1 v/v, pH 8.9) during 20 min at 30ºC, dried in a Speedvac evaporator, treated with 5 ul of 200 mM (pH 8.9) ammonium carbonate plus Tween 20 (0.02% v/v) and then digested with 2 ul TPCK-trypsin (0.25 mg/ml in 200 mM ammonium carbonate pH 8.9).

After digestion, tryptic peptides were separated by reverse-phase High-Perfomance Liquid Chromatography (HPLC) (Applied Biosystem Model 140A, Foster City, CA, USA) on a Brownlee C18 column (220×2.1 mm) equilibrated with 5% (v/v) acetonitrile, 0.1% TFA in water. Elution was performed at a flow rate of 0.2 ml/min with a 8.48% acetonitrile 0.1% TFA in water (v/v) linear gradient in 70 min. Selected peptides were applied to a polybrene-coated glass filter and sequenced in an Applied Biosystems Model 477 A Automatic Sequencer run according to the manufacturer's instructions.

RESULTS

Fractionation process of the bovine seminal plasma

The bovine seminal plasma (BSP), from a pool of semen samples, after liquefaction (natural proteolysis) and separation of material of molecular weight lower than ∼10 kDa (dialysis) was submitted to chromatography (Fig. 1). The Con A-Sepharose column yielded 92% of the starting material when 1200 Da cut-off dialysis bags were employed in the isolation of the fractions but only 25% when the same dialysis were made using M_r 12000 Da cut-off tubing, showing that the unfractionated seminal plasma contained major amounts of low-molecular weight products in spite of the previous dialysis carried out during its preparation (see Materials and Methods and Fig. 1). The Con A "non-interacting" (yield 77.5%), "weakly-interacting" (yield 9%) and "strongly-interacting" (yield 13.5%) fractions of bovine seminal plasma (fractions 1, 2 and 3) (Fig. 1) were used for UV-MALDI-TOF MS analysis together with fraction II obtained from fraction 1 ("non-interacting" material) through gel permeation and further *Phaseolus vulgaris* leucoagglutinin (L-PHA)-agarose chromatography (Fig. 1). It is worth of note that due to the fractionation, the (glyco) proteins of fractions 1, 2 and 3 were submitted to the UV-MALDI MS analysis in concentrations 30%, 11 times and 7–8 times higher than in the original seminal plasma. Some of the (glyco)proteins of fraction II were also characterized after FPLC fractionation and SDS-PAGE of its subfractions, coupled to "in gel" digestion and microsequencing.

UV-MALDI-TOF mass spectrometry

Matrices

UV-MALDI-TOF MS analysis was conducted in comparative way by using different UV-MALDI matrices (Tables 1 and 2). Some experiments were performed combining two matrices (Table 2). Two sample preparation methods were used (Methods A and B). The resulting solid partially crystalline layers analyte-matrix were found to be relative homogeneous in all the cases (Fig. 2). Sinapinic acid (SA) and Harmaline (Ha) as matrices showed signals of higher quality in positive and negative ion modes using Method A (Table 1). Gentisic acid (GA) and trans α-ciano-4-hydroxycinnamic acid (CHC) showed signals of good quality in positive ion mode by using Method A (Table 1). Nor-harmane (nor-Ho) showed signals of good quality in positive ion mode and low intensity signals in negative ion mode. 2,4,6-Trihydroxyacetophenone (THAP) showed signals of low quality only in positive ion mode by using Method A (Table 1). 5-Chloro-2-

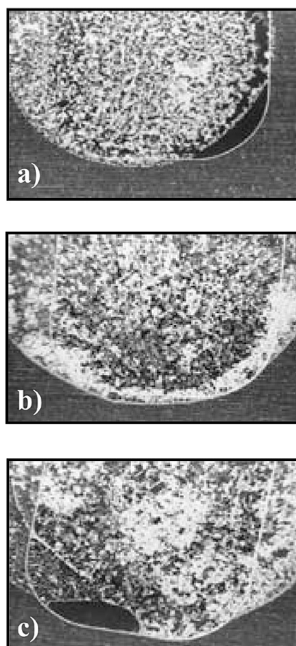

Fig. 2 Matrices: a) Sinapinic acid (SA), b) Sinapinis acid plus fraction 1 ("non-interacting fraction"), c) Sinapinic acid plus fraction 2 ("weakly-interacting fraction").

mercaptobenzothiazole (CMBT) did not show any signal in positive and negative ion modes by using Method A (Table 1). By using the sandwich method and combining different matrices (Table 2), the best results were obtained with the combination GA: A: SA: SA (A, analyte). By using Method B, only SA and Ha matrices showed signals of low quality in positive and negative ion modes. Thus, the results shown and discussed are those obtained using SA as matrix and the sample preparation method A (Table 1) (mixture of analyte solution and matrix solution 1:4 v/v ratio, in the optimum experimental conditions).

Spectra

The intensity of the peaks was low and the spectra were obtained with different sensitiviness from 10 mV = 100% to 0.1 mV = 100%. According to the sensitiviness of the spectrum only peaks above a threshold were considered. No PSD or reflecton mode experiments were carried out due to the low intensity of the peaks.The spectra obtained in linear positive and negative ion modes in the 4.0–90.0 kDa mass/charge range showed well defined zones, namely: 5.5–8.0 kDa, 10.0–12.0 kDa, 12.5–14.0 kDa, 23.2–23.7 kDa, 26.1–28.0 kDa (not in sample 3) and 38.0–40.0 kDa. Very small and ill-definedl peaks in the higher sensitivity spectra were seen up to 90.0 kDa. The most intense and defined peaks appeared at low molecular weights but when the molecular weights increase those qualities sharply diminished. Peaks from the matrix appeared with higher intensity at mass/charges lower than 5.0 kDa but due to the low intensity of the (glyco)protein signals they were seen all over the spectrum. Small amounts of low molecular weight (glyco)peptides were lost undifferentiated from the matrix. As all the peaks observed in the negative -ion mode appear also in the positive-ion mode, together with another few ones, the UV-MALDI MS analysis was carried out using the positive-ion mode and only those peaks clearly distinguishable from the matrix baseline were taken into account. The spectra were not quantitatively reproducible and the relationship intensity of the peak/amount of (glyco)protein was only qualitative. The mass/charge ratio is given in Da for peaks in the range 5.5–20.0 kDa and in kDa for small ill-defined peaks in the range 20.0–

90.0 kDa (spectra not shown), with about 0.1–0.2% error inherent to the method; besides owing to the complex character of the (glyco)protein mixtures analyzed, partial superposition of the signals occur, resulting in a minor shift of the m/z value corresponding to the maximun intensity of each signal. Several spectra were obtained for each fraction, those spectra shown in Figs 3–6 were considered representative but a few m.w. mentioned in the text were obtained from others.The energy of the UV laser is higher than those stabilizing the protein complexes and, as a consequence, the spectra should show mainly monomeric protein units (Fenselau, 1997). This is consistent with the fact that in all the fractions the major components appear with molecular weights in the ranges 5.5–8.0 kDa and 12.5–14.0 kDa.

UV-MALDI-TOF MS data

Con-A "non interacting" material (fraction 1)

The simplicity of the spectrum (Fig. 3a, 7.8 mV＝100%) compared to those of the other fractions (see later), does not represent the composition of the bovine seminal plasma but, possible, the difference in concentrations between the (glyco)proteins. Expansion of the m/z scale (11.5–15.0 kDa) (Fig. 3 b, 100%＝5.9 mV, only peaks higher than 10% were considered) enlarge the major peaks but it does not fractionated them. In 12.5–14.0 kDa range, appears two major peaks at 12801 and 13464 Da, and six minor ones at 12985, 13179, 13372, 13607, 13680 and 13871 Da. In zone 5.0–8.0 kDa (Fig. 3 c, 100%＝1.9 mV, only peaks higher than 40% were considered), it shows major peaks at 6401 and 6725 Da togther with others at 6156, 6596, 6821 and 8000 Da (the last one not seen in Fig. 3) and several peaks crowded between 5450–5820 Da.

Spectra in the 20–40 kDa zone (not shown) obtained with higher sensitivity (0.9 mV＝100%) showed small, hardly differenciated from the baseline, peaks at 20.08, 20.57, 26.19, 26.41, 26.82, 27.15 and 40.00 kDa. No other peaks were found up to 90.0 kDa.

Con-A "weakly interacting" material (fraction 2)

In fractions 2, 3 and II (Figs. 4, 5 and 6), the complexity of the spectra is higher than in fraction1 due to the previous elimination of most of the above mentioned major proteins and consequently the higher concentration of minor components of the seminal fluid. Only peaks higher than 25% were considered in spectra 4a (100%＝5.2 mV) and b (100%＝5.1 mV) of fraction 2; 5527, 5710, 6150, 6485, 6630, 6796, 7881 and 8190 Da weak peaks were found in the ranges 5.5–8.5 kDa, and others (11785 Da) in the range 10.0–12.0 kDa and 15–18.1 kDa (14191 and 16942 Da). Major and complex peaks are better appreciated in spectrum 4b with m/z 12756, 12915, 13044, 13113, 13391, 13481 and 13750 Da. Small and ill-defined peaks (spectra not shown) were found centered at 16.94, 20.53, 26.93, 33.23, 40.12, 41.11, 46.65, 52.16 and 56.09 kDa. No other peaks were found up to 90.0 kDa.

Con-A "strongly interacting" material (fraction 3)

The spectrum of fraction 3 (Fig. 5a), carried out with sensitivity 2.5 mV＝100% (only those peaks higher than 10% were considered), in the mass/charge range 6–18 kDa showed minor peaks at 6079, 6300, 6410, 6500, 6610, 6700 and 7099 Da. Major and complex peaks were seen at 12810, 12963, 13177, 13243, 13342, 13446, and 13656 Da. The lower m. w. zone was scanned in a expanded scale (4–8 kDa) (Fig. 5c) and with higher sensitivity (1.2 mV＝100%) (only peaks higher than 60% were used). Peaks were found at 5423, 5698, 5800, 5890, 5991, 6075, 6186, 6210, 6280, 6398, 6500, 6590, 6688 and 7099 Da.

The zone 12–15 kDa (Fig. 5b) was scanned with a sensitivity 1.3 mV＝100% and showed major and complex peaks at (only those higher than 20% were considered) 12730, 12800, 12835, 12850, 12875, 12905, 12923, 12950, 12993, 13006, 13038, 13095, 13145, 13152, 13177, 13248, 13261, 13305, 13340, 13365, 13375, 13405, 13435, 13446, 13472, 13515, 13556, 13626, 13650, 13675, 13695, 13790, 13850, 13900 and 13940 Da.

Spectra (not shown) obtained in the 18–50 kDa and 50–90 kDa scales and with the highest intensity (～0.1 mV＝100%) showed peaks (only those higher than 60% were considered) centered

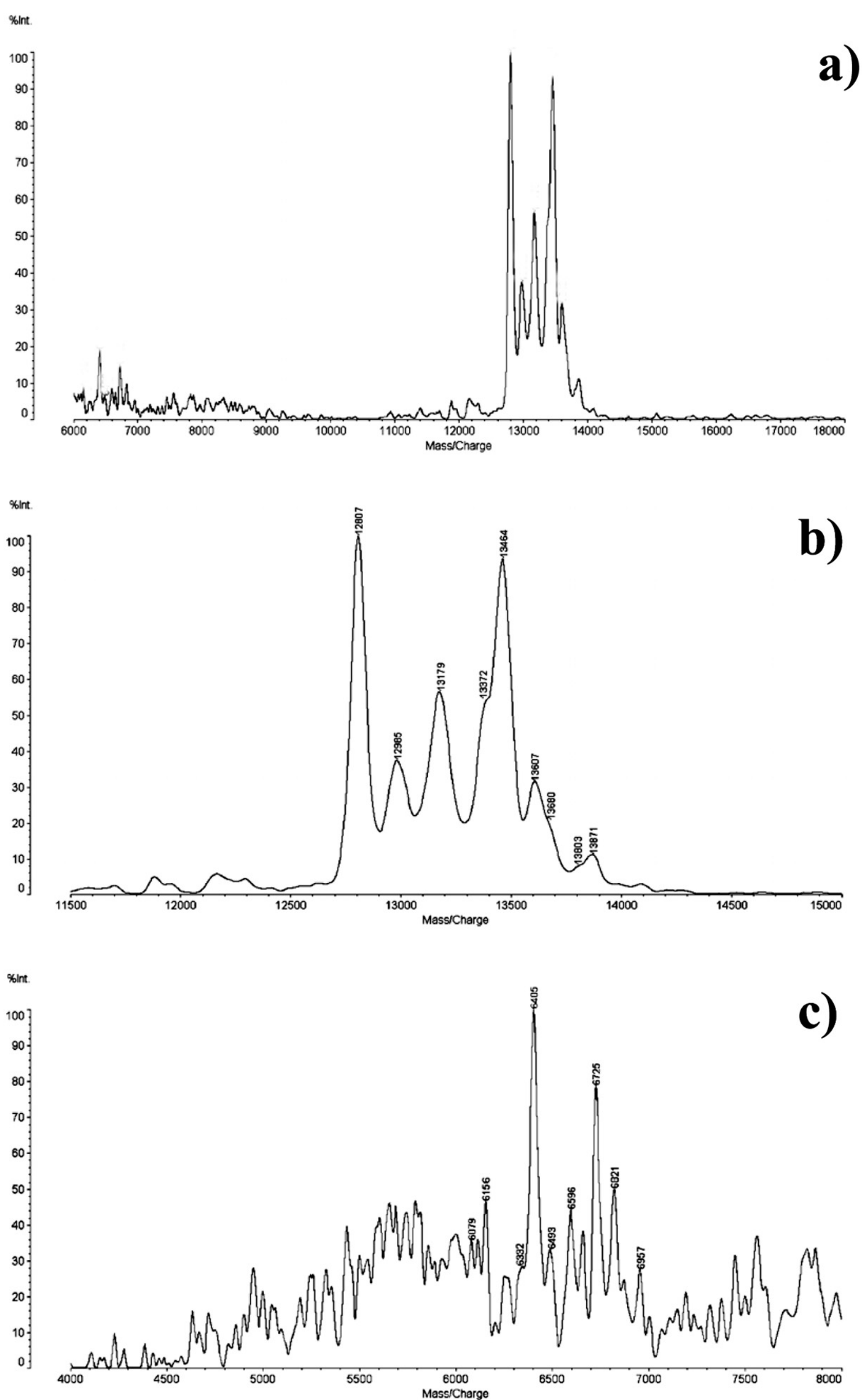

Fig. 3 UV-MALDI-TOF MS of the “non-interacting” fraction (Fraction 1). a) Scale 6.0–18.0 kDa, sensitivity 100%＝7.8 mV, the m.w. are given in the spectra with the expanded scales; b) Scale 11.5–15.0 kDa, sensitivity 100%＝5.9 mV. The six peaks seen in Fig 3a) are expanded but no new peaks are observed. c) Scale 4.0–8.0 kDa, sensitivity.

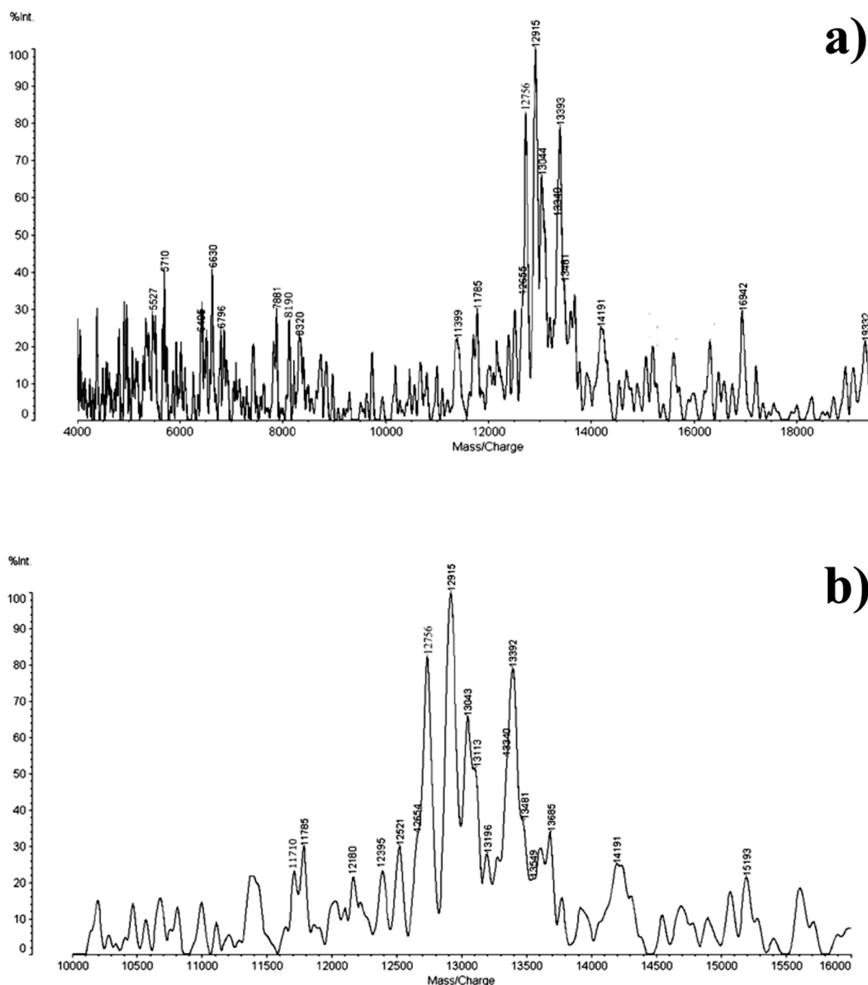

Fig. 4 UV-MALDI-TOF MS of the "weakly interacting" fraction (fraction 2). a) Scale 4.0–19.5 kDa, sensitivity 100% = 5.2 mV. Only those peaks higher than 25% and mass/charge higher than 5.5 kDa were considered. b) Scale 10.0–16.0 kDa, sensitivity 100% = 5.1 mV. Only those peaks with sensitivity higher than 25% were considered.

at 18.45, 20.00, 22.75, 26.55, 30.00, 32.50, 32.80, 36.20, 36.70, 37.95, and 46.90 kDa. Also at 55.0–55.1, 60.0–65.0, 69.0–70.0, 74.0 and 84.0–85.0 kDa.

Con-A "non-interacting" material, after further fractionation through gel-permeation and Phaseolus vulgaris leucoagglutinin (L-PHA)-agarose chromatographies (Fraction II)

The spectra of fraction II (Fig. 6a) carried out with a sensitivity 6.8 mV = 100%, in the mass/charge range 4–16 kDa showed in the zone 5–7.4 kDa (only those peaks higher than 10% were considered) minor but complex peaks between 6.4–6.8 kDa (6535, 6688 and 6755 Da). Major and also complex peaks were observed between 10.0–14.5 kDa, namely: 10133, 12735, 12907, 12985, 13465, 13520, and 13702 Da. Expansion of the scale (5.0–7.4 kDa) and higher sensitivity (0.9 mV = 100%) (Fig. 6b) showed minor peaks (only those higher than 30% were considered) at 6385, 6457, 6510, 6540, 6668, 6755, 6781, 6842, and 6947 Da.

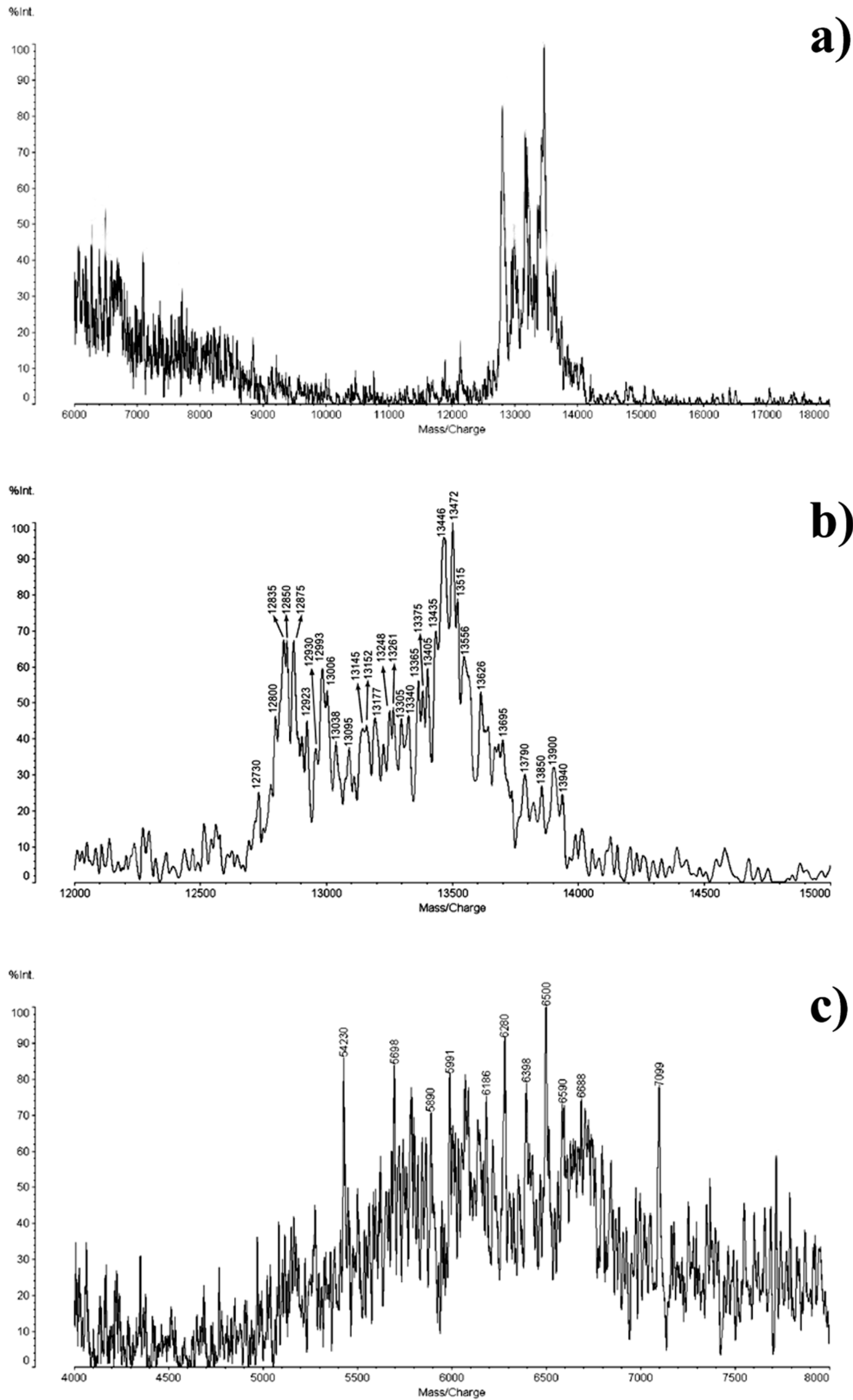

Fig. 5 UV-MALDI-TOF MS of the "strongly interacting" fraction (Fraction 3). a) Scale 6.0–18.0 kDa, sensitivity 100% = 1.3 mV. Only those peaks higher than 15% were considered, the m.w. are given in the spectra with the expanded scales. b) Scale 4.0–8.0 kDa, sensitivity 100% = 0.7 mV. Only those peaks higher than 50% and mass/charge higher than 5.5 kDa were considered. c) Scale 12.0–15.0 kDa, sensitivity 100% = 1.2 mV. Only those peaks higher than 20% were considered.

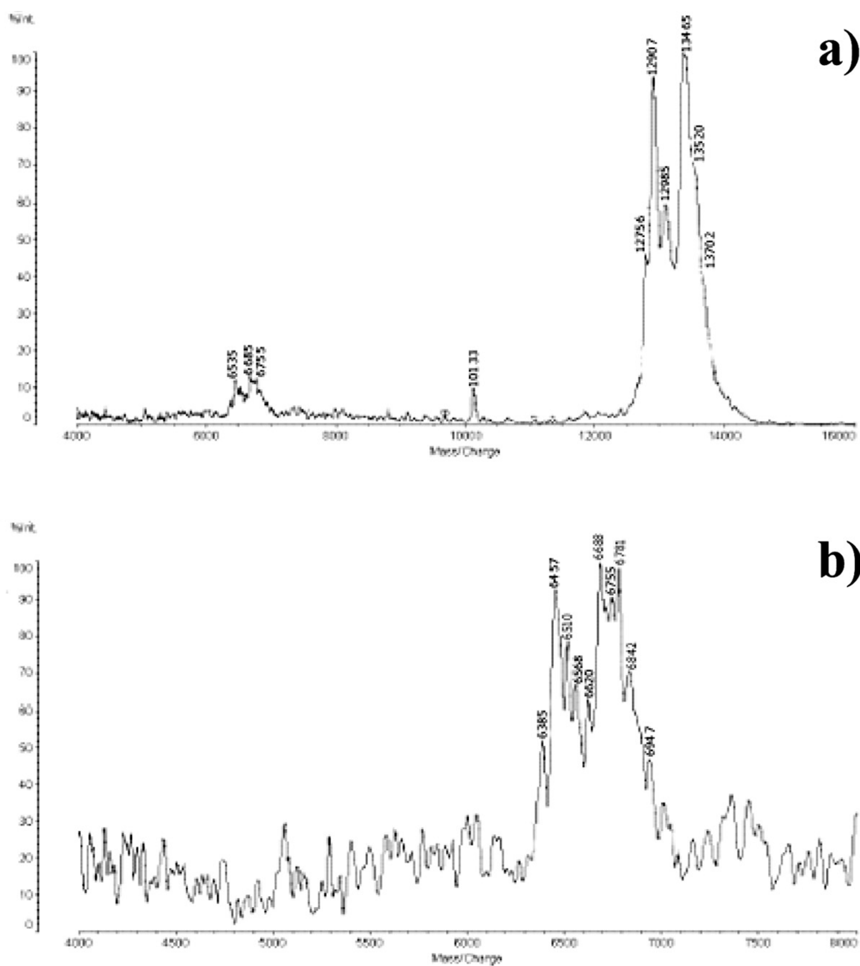

Fig. 6 UV-MALDI-TOF MS of fraction II. a) Scale 4.0–16.0 kDa, sensitivity 100% = 6.8 mV. Only those peaks higher than 5% were considered. b) Scale 5.0–7.4 kDa, sensitivity 100% = 0.9 mV. Only those peaks higher than 40% were considered.

SDS-PAGE-sequencing studies

Chromatographic studies Fast protein liquid chromatography (FPLC)

The FPLC purification of fraction II yielded 3 fractions IIa, IIb and IIc and a small shoulder IIa′ (Fig. 7). IIa, the major one, appears at the void volume, II′a is situated about 50 kDa and IIb and IIc in the 45 kDa and 29 kDa range, respectively.

Analysis by electrophoresis and microsequencing studies

In the SDS-PAGE analysis (not shown) fraction II shows major bands of 14,18, 20 and 24 kDa and many minor bands between 24 and 66 kDa. Fraction IIa (void volume) showed six major bands 14, 18, 24, 40, 60 and 66 kDa. The shoulder IIa′ only showed two major bands 14 and 18 kDa (the latter seems to be two bands closed together) and a minor band of 66 kDa. IIb and IIc also showed two major bands at 14 and 18 kDa. The "in gel" digestion of the 18 kDa band of IIa′, fractionation of the peptides mixture and sequencing of one of them gave the sequence HSNLIESQENS. This sequence shows 100% homology with the portion corresponding to residues 210–220 of the amino acid sequence of osteopontin, acces code P 31098 (Cancel et al., 1999). Other peptide analyzed gave the sequence VSXVTPXF. This sequence corresponds to the C

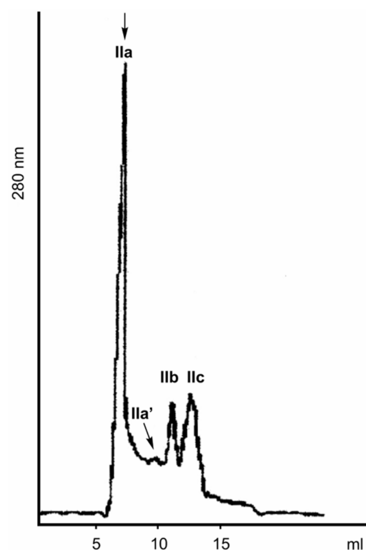

Fig. 7 FPLC-Superose chromatography of fraction II.

terminal (portion 97–104) of the HE4 (glyco)protein, access code Q 14508 (Kirchhoff et al., 1991; Kirchhoff et al., 1996; Kirchhoff et al., 1998).

The "in gel" digestion of the band 18 kDa of IIb and microsequencing of one the peptides obtained by HPLC fractionation gave the sequence DXQLSEDNVILPK. This sequence shows 100% homology with the amino-terminal end (residues 1–13) of bovine seminal protein (BSP-A3) access code P04557 (Calvete et al., 1996; Manjunath and Sairam, 1987; Nauc and Manjunath, 2000). The sequences obtained by microsequencing of two peptides from the 14 kDa band of the same fraction were: MDWLPR and NTNXGGILKEESG. These sequences show 100% homology with the portions corresponding to residues 21–26 and 27–39 respectively of the amino acid sequence of αSFP protein access code P29392 (Calvete et al., 1996).

The microsequencing of three peptides from the higher molecular weight band (18 kDa) of IIc yielded the following sequences QDEGVXTEPT, VFPF and SMWMS. This sequences show 100% homology with the portions corresponding to residues 2–11, 25–28 or 70–73 and 88–92 respectively of the aminoacid sequence of the bovine seminal plasma glycoproteins BSP-A1 and BSP A2 (collectively named PDC-109) access code G 134452 (Calvete et al., 1999). On the other hand the microsequencing of one of the peptides of the lower molecular weight band gave the sequence MNWLP. This sequence shows 90% homology with the portion corresponding to residues 21–25 of αSFP protein (Calvete et al., 1996).

DISCUSSION

Seminal plasma is a complex fluid containing a mixture of (glyco)proteins, originated from the testis, the epidiymis and from the accessory glands, involved in different steps of fertilization. (Glyco)proteins that participate in sperm-egg recognition, modulation of capacitation of the spermatozoon, acrosome reaction, etc, were described in these fluids as well as fertility-associate proteins (Calvete et al., 1996; Killian et al., 1993; Thérien et al., 1995; Iborra et al., 1996). Some of them in the bovine seminal plasma have been considered markers associated with bull fertility (Killian et al., 1993).

The methodology mostly used to analyze the complex mixtures of (glyco)proteins of seminal

plasmas has been the SDS-reducing D- and 2D-PAGE. A two dimensional map of seminal plasma from a fertile man allowed the detection of about 750 spots which shows the chemical and physico-chemical complexity of this fluid (Starita-Geribaldi et al., 2001). Nevertheless, in spite of the resolving capacity of the SDS-PAGE techniques they showed many drawbacks in the analysis of seminal plasma which were inherent to the methodology and/or to the complexity of the plasma itself, namely:

a) The SDS-PAGE separation is only the first step in the identification of the proteins, which needs further trypsic lysis, fractionation of the peptide mixtures and sequence analysis of some of the peptides, or the same trypsic procedure coupled to the determination of the UV-MALDI MS peptide mass fingerprint further analyzed with the appropriate software (Silva et al., 2003).

b) The self-aggregation as well as the formation of complexes with other proteins usually found in seminal fluids, indicates that there is not a clear correspondence spot/substance in the SDS-PAGE analysis (Calvete et al., 1996; Manjunath and Sairam, 1987).

c) The SDS-PAGE only permits determination of the ranges of molecular masses and it is not discriminant between low-molecular weight (glyco)proteins (Fung et al., 2004; Marquinez et al., 2000) as those found in seminal plasmas (Calvete et al., 1996).

d) The inability to adequate resolve basic proteins is a known shortcoming of 2D E (Fung et al., 2004).

e) The acid nature of a protein could affects its mobility in SDS-PAGE (Denhardt and Guo, 1993).

Previous positive-ion mode UV-MALDI experiments with isolated (glyco)proteins of bovine seminal plasma (Calvete et al., 1996) and seminal fluids from other species (Focatelli et al., 1997; Teixeira et al., 2002) show the detection of a unique major ion $(M+H)^{+}$ corresponding to each single compound together with, in some cases, of small peaks of $(M+2H)^{++}$ and/or $(2M+H)^{+}$ (Focatelli et al., 1997). This correspondence major ion/compound and its sensitivity make the UV-MALDI-TOF MS an useful technique for the (glyco)protein analysis of seminal fluids in spite of its complexity, and for the direct identification of its constituents. Tentative identification can be made using the approximate SDS-PAGE lower molecular weight (see later) as they are in the same range, even if somewhat higher, than those determined by UV-MALDI-MS (Manjunath and Sairam, 1987). It is worth of note that the molecular masses of the major ions $(M+H)^{+}$ of the bovine seminal plasma (glyco)proteins are in the range 12–14 kDa while showing much higher m. w. in SDS-PAGE (Calvete et al., 1996), indicating a process of disaggregation of the (glyco)protein complexes previous to the volatilization/ionization step. This is consistent with the high energy of the UV-laser used in the MALDI experiment (Fenselau, 1997).

UV-MALDI-MS can be used for the characterization of (glyco)proteins in mixtures in two different ways, namely: a) through the determination of the MALDI molecular masses in the sample which identification suppose its previous knowledge or the use of standard or its selection by approximation using the lower SDS-PAGE molecular weight, and b) the separation of the (glyco)proteins by SDS-PAGE and further “in gel” digestion. The peptide mass fingerprint of the digest obtained through UV-MALDI-MS submitted to an appropriate software allows the characterization of the protein (Overberg et al., 1990; Fung et al., 2004).

Large-scale isolation of major bovine seminal plasma (glyco)proteins (Calvete et al., 1996) permitted the UV-MALDI-MS molecular weight determinations of the acidic seminal fluid protein (αSFP: PAGE m.w. 14 kDa, UV-MALDI-MS m.w. 12923 Da (Calvete et al., 1996)), bovine seminal protein (BSP-A3: PAGE m.w. 18 kDa, UV-MALDI-MS m.w. 12789 Da (Calvete et al., 1996)), the glycoforms of BSP-A3 usually denominated PDC-109 (BSP-A1 plus BSP-A2, PAGE m.w. 13–14 kDa, UV-MALDI-MS average m.w. 13444 Da (Calvete et al., 1996)) and the tissue inhibitor of metalloprotease 2 (TIMP-2: UV-MALD-MS m.w. 21707 Da (Calvete et al., 1996)). Direct

analysis of the unfractionated human seminal fluid by UV-MALDI MS revealed the presence of over 30 peptide components in the mass/range 5–10 kDa. Due to the signal suppression effects inherent to the UV-MALDI process, components greater than 10 kDa were not supposed to be detected in the presence of major amounts the low-mass peptides (Overberg et al., 1990). As the peptide mass maps were collected in reflectron mode it is possible that some components with masses higher than 10 kDa were not detected due to its low concentrations respect to that of the peptides. A sperm adhesin structurally homologous to sperm adhesins from boar and stallion was isolated from buck seminal plasma and shown by UV-MALDI-MS to have molecular mass of 12500 Da (Teixeira et al., 2002). The peptide mass fingerprint technique was used to identify in human seminal plasma the prostatic acid phosphatase (PAP) and prostate specific antigen (PSA) from SDS-PAGE spots appearing in the ranges 44.4–46.9 kDa and 30.2–31.8 kDa, respectively (Fung et al., 2004). First spectra (not shown) carried out with dialyzed seminal plasma indicate that most of the major (glyco)proteins appear in the range 12.5–14.0 kDa, crowding that zone and suggesting the convenience of a previous fractionation that not only would simplify the spectra but also would concentrate minor fractions. Fractionation of seminal plasmas has been attempted using different techniques (Calvete et al., 1996) but affinity chromatography has the advantage to be preparative, facilitating through the specificity of the binding substance the identification of the peaks (i.e. use of lectins for glycoproteins) (Marquinez et al., 2000) or the isolation of a specific product from a complex mixture (i.e. heparin in the isolation of TIMP-2) (Calvete et al., 1996). The use of Concanavalin-A (Con-A) lectin as the binding substance in the affinity chromatography of human (Marquinez et al., 2003) and bovine seminal plasma (Marquinez et al., 2000) has been previously reported. It was based in the specificity of the lectin to interact with some N-linked oligosaccharides-containing glycoproteins, fractionating the seminal (glyco)proteins into "non-", "weakly-" and "strongly-interacting" fractions (Marquinez et al., 2000). For these reasons the seminal plasma from a pool of semen samples, after elimination of most of the low-molecular weight material (Overberg et al., 1990), was previously submitted to Con A-Sepharose chromatography-fractionation. The very low total yield of the Con A-Sepharose column (25%) when the M_r 12000 cut-off tubes were used for dialysis of the fractions, demonstrates the presence of high percentages of low-molecular weight compounds in the starting sample in spite of the previous dialysis during the preparation of the seminal plasma. The chromatography yielded three fractions, namely: a major one (fraction 1, 77.5% yield) with proteins and some glycoproteins which do not interact with the lectin and two minor ones; fraction 2 (9% yield) constituted by glycoproteins which interact only weakly with the lectin, and fraction 3 (13.5% yield) with glycoproteins, which interact strongly with the lectin. Further fractionation of the "non-interacting" material (fraction 1) by G.P.C. in Sephadex G-50 and L-PHA agarose chromatography (Fig. 1) yielded fraction II.

When these four fractions of bovine seminal plasma were submitted to UV-MALDI analysis, peaks appearing at mass/charge higher than 20 kDa were weak and some times hardly differentiated from the baseline. It is not known whether this is due to the suppression effects of the lower molecular weight compounds (Overberg et al., 1990), to the low concentration of these compounds in the seminal plasma or to the fact that they are the remainder of complexes mostly dissociated before the volatilization/ionization step.

Minor peaks in the spectra of fraction 1 (Fig. 3a, b and c), in the lower molecular weight range (5.5–8.0 kDa) are double-charged ions (6405 and 6725 Da) of the bovine seminal plasma (BSP-A3) and of the PDC-109 glycoproteins, together with peaks in the 5400–5900 Da and 6100–7000 Da ranges (Fig. 3c) that could correspond to caltrin (PAGE m.w. 6500 Da) (Kameswari and Prasad, 1994; Lewis et al., 1985; Sitaram, 1986; Clark et al., 1993) and/or any other positive modulating factor, with molecular weights in the same range (Clark et al., 1993). Caltrin and the other peptides are found in all the samples in spite of the two dialysis carried out during the preparation and fractionation of the seminal plasma with bags of cut off 12000 Da., suggesting that these hydrophobic

peptides are not free in the seminal plasma but complexed in aggregates of higher molecular weights. This hydrophobycity explain why some of them are retained in the Con A (fractions 2 and 3) and is consistent with the existence of a hydrophobic pocked in the lectin (Goldstein and Hayes, 1978; Edelman and Wang, 1978). The 8000 Da peak (not shown in Fig. 3) could correspond to the calcium transport inhibitor protein with apparent molecular weight 9.6–10.5 kDa (g.p.c. and SDS-PAGE), responsible for delaying uptake of calcium into ejaculated sperm (Ruffo et al., 1982).

The spectra of fraction 1 (Fig. 3a and b) show two major peaks (12801 and 13464 Da) which form double ions (see above) and that were recognized as bovine seminal proteins (BSP-A3) and BSP-A1 (Calvete et al., 1996). The small peak at 10167 Da (only sporadically found in this fraction, not shown in Fig. 3) corresponds to the human epididymis-specific (putative) glycoprotein (HE4) of low molecular weight (10 kDa) (Kirchhoff et al., 1991) latter found in bovine seminal plasma, which dissociates from ejaculated sperm during "in vitro" capacitation (Kirchhoff et al., 1991; Kirchhoff et al., 1991). Similarities between HE4 and a group of small cysteine-rich secretory polypeptides which are known to participate specifically in protein-protein interactions (Kirchhoff et al., 1991) were demostrated. The PDC-109 glycoproteins, BSP-A1 and BSP-A2, belong to the BSP family having identical amino acid sequence and differ only in the degree of glycosylation (Manjunath and Sairam, 1987). They are usually found together and sometimes were considered as a single chemical entity (PCD-109) (Manjunath and Sairam, 1987). Both glycoproteins have been reported containing galactosamine and sialic acid but neutral sugars were supposed to be present only in BSP-A1. It was speculated that the carbohydrate was linked to the protein in the form of O-glycosidic linkages (Manjunath and Sairam, 1987). The presence of N-acetyl neuraminic acid is compatible with the fact that over 90% of the BSP-A1 bound to wheat-germ lectin-Sepharose and that the glycoprotein could only be eluted from the lectin by N-acetyl glucosamine (Manjunath and Sairam, 1987; Greenaway and LeVine, 1973; Calvete et al., 1994; Gerwig et al., 1995). The parent protein BSP-A3 and several glycoforms, three of them previously unreported (Table 3), were present in the spectra of fraction 3 and subfraction II (Figs. 5 and 6).

The small peak at 21.8 kDa (not shown) could correspond to the non-glycosylated inhibitor of metalloproteinase 2 (TIMP-2) (UV-MALDI m.w. 21707 Da) (Calvete et al., 1996; De Clerk et al., 1989). TIMP-2 is a secretory protein of bovine aortic endothelial cells and was isolated from bovine seminal plasma by affinity chromatography on Heparin-Sepharose (Calvete et al., 1996). Prostate specific antigen, seminal ribonuclease, fertility-associate antigen (FAA) and BSP-30K have SDS-PAGE m.w. in the range (SDS-PAGE 28–30 kDa) and could corespond to the 26.19–

Table 3 Molecular weights and possible monosaccharide compositions of the oligosaccharide chains of glycoproteins of the BSP family.

M. w. of the glycoproteins	Name of the (glyco) protein	M. w. of the olygosaccharide chain		Composition of the oligosaccharide chains			
Da[x]		Exp.	Calc. Da[u]	N-acetyl galactosamine	i-galactose[z]	e-galactose[y]	Neuraminic acid
12800[w]	BSP-A3						
13177	unknown	377	382	1		1	
13342	BSP-A2[v]	542	551	1			1(N-acetyl, O-acetyl)
13465	BSP-A1[v]	665	671	1	1		1(N-acetyl)
13520	unknown	720	706	1	2	1	
13650	unknown	850	850	1	1	1	1(N-acetyl)

[z] Internal galactose, [y] external galactose, non-reducing end-chain galactose. [x] The molecular weights are affected by about 0.1–0.2% error inherent to the method but also by a minor shift of the m/z value owing to the partial superposition of the signals. [w] Peaks appearing in the different fractions. [v] The composition of the oligosaccharide chains calculated correspond exactly to those determined experimentally (10,39,40). [u] The m. w. of the oligosaccharide chains is obtained as the difference between the m.w. of the supposed glycoproteins and that of the protein.

27.15 kDa peaks. PSA, a 29–33 kDa protein produced and secreted principally by the prostate epithelium and epithelium lining of the periurethral gland, is the primary biomarker for prostate cancer (Mettlin et al., 1996). Bull is the only mammal known to produce a seminal ribonuclease. Its physiological role is unknown but it displays antispermatogenic, antitumor and immunosuppressive activities (Kim et al., 1995; Mastronicola et al., 1995). BSP-30K belongs to the BSP-family and stoichiometrically associate with PDC-109 to form a high m.w. complex (Calvete et al., 1996). The peak at about 40 kDa (not shown) could be prostatic acid phosphatase (PAP) (SDS-PAGE m.w. 44.4–46.9 kDa) (Marquinez et al., 2003). Both, aSFP and PDC-109 have been previously identified by the SDS-PAGE sequencing technique in subfractions Ib and III of sample 1, respectively (Fig. 1) (Marquinez et al., 2000).

All the products identified in sample 1 were proteins or O-linked glycoproteins which do not interact with the lectin except HE4. This is a putative glycoprotein with only one possible carbohydrate chain of the N-type but of unknown structure, which only sporadically appears in the spectra of the "non-interacting" material (fraction 1) possible due to its low concentration relative to those of the major proteins BSP-A3 and BSP-A1, but that is clearly visible in the spectra of fraction II.

The spectra of fraction 2 (Fig. 4a and b) showed small peaks in the mass/charge zone 5500–8200 Da (Fig. 4a), at 11785 Da and in the mass/charge zone 14000–17000 Da (Fig. 4a and b) and major ones at 12756, 12915, 13044, 13114, 13392, 13481 and 13750 Da. The peak at 12915 Da is assigned to acid seminal fluid protein (aSFP, fraction 1: 12930 Da). Other major peaks could be considered as a group of a protein and its glycoforms (Table 4). Thus, if the compound with mass/charge 12756 Da is a protein, other molecular weights could be arranged in a sequence (Table 4) differing in units of a N-acetyl glycosamine, interior and/or non-reducing end-chain neutral hexoses and neuraminic acids (Table 4). Spectra in the 20–60 kDa scale (not shown) showed small peaks at 40.12, 41.11 kDa (PAP?, isoform of OPN?), 52.16 and 56.01 kDa (OPN?), hardly differentiate from the baseline.

The spectra of fraction 3 (Fig. 5a, b and c) as those of fraction 2 (Fig. 4) showed several peaks in the range 5.0–7.5 kDa confirming that, in spite of the repeated dialysis, several peptides were retained in the starting sample. Spectra obtained with higher sensitivity and in an expanded mass/charge scale (12.5–14.0 kDa) (Fig. 5b) showed an enormous complexity with more than 32 peaks between which aSFP (12923 Da) was recognized together with the enlarged BSP-family (Table 3) with the PDC-109 peak resolved into three peaks corresponding to the glycoforms (13340 Da and 13644 Da) and to the average mass (13446 Da) (Fig. 5a), and another new family starting from a

Table 4 Molecular weights and its interpretation as corresponding to a family of a protein (12756 Da) and its glycoforms.

M. w. of the glycoproteins	M. w. of the olygosaccharide chain		Composition of the oligosaccharide chains			
Da[x]	Exp.	Calc. Da[v]	N-acetyl glycosamine	i-hexose[z]	e-hexose[y]	Neuraminic acid
12756[w]	—					
13134	378	382	1		1	
13305	549	544	1	1	1	
13472	716	713	1	1		1(N-acetyl), (O-acetyl)
13595	839	833	1	2		1(N-acetyl)
13790	1034	1037	1	3		1(N-acetyl), (O-acetyl)

[z] Internal hexose, [y] external hexose, non-reducing end-chain hexose. [x] The molecular weights are affected by about 0.1–0.2% error inherent to the method but also by a minor shift of the m/z value owing to the partial superposition of the signals. [w] Peaks appearing in the different fractions. [v] The m.w. of the oligosaccharide chains is obtained as the difference between the m.w. of the supposed glycoproteins and that of the protein.

protein of 12730 Da and glycoforms at 13248 Da, 13405 Da, 13446 Da and 13556 Da (Table 5). As in the previous cases the member of this new family contain only one oligosaccharide chain differing in the content of a N-acetyl glycosamine, internal and/or terminal hexoses or sialic acids.

Most of these products in fractions 2 and 3 are proteins and glycoproteins with O-type oligosaccharides which should not be retained by the Con A lectin. These facts suggests that the fractionation was not due to specific affinity interactions of the carbohydrate chains of the glycoproteins with the lectin but, possible, to non-specific hydrophobic interactions of the proteinic part with the hydrophobic pocked of the Con A (Goldstein and Hayes, 1978; Edelman and Wang, 1978). The composition of the O-linked oligosaccharides shown in Tables 3, 4 and 5 are in complete agreement with published data for the oligosaccharides of BSP-A1 and BSP-A2 (Manjunath and Sairam, 1987; Calvete et al., 1994; Gerwig et al., 1995).

Also major peaks but of lesser intensity (Fig. 5c) were a triplet at 12945, 12993 and 13006 Da and a quadriplet at 13145 13152, 13195 and 13248 Da, which are not glycoforms according to the small differences in their molecular weights. Minor peaks at 13626 Da and 13850 Da also appear. In the low-molecular weight range (Fig. 5b) peaks corresponding to double ions of 12810 Da and 13464 Da are seen together with others. Considering that the molecular masses determined by UV-MALDI-MS are in the same range, but always lower, than those minimal obtained by SDS-PAGE, the peaks at about 21–22 kDa (not shown) would correspond to the tissue inhibitor of metalloproteinase 2 (TIMP-2) (De Clerk et al., 1989), the low-intensity peak at about 27 kDa (not shown) would correspond to the prostate specific antigen (PSA) (Mettlin et al., 1996), seminal ribonuclease (Kim et al., 1995; Mastronicola et al., 1995) or BSP-30K (Manjunath and Sairam, 1987), while that at 40 kDa would correspond to the prostatic acid phosphatase (PAP) (Marquinez et al., 2003) and/or to an isoform of OPN. Those small peaks in the range 55.0–55.1 (OPN?) (Denhardt and Guo, 1993), 60.0–65.0, 69.0–70.0 and about 77.0 kDa (not shown) could correspond to aggregation complexes or less proteolyzed native high molecular weight (glyco)proteins.

The UV-MALDI-TOF MS- and SDS-PAGE-sequencing analysis of the low-molecular weight (glyco)proteins of fraction II of bovine seminal plasma

Only those bands at 13.0–14 kDa and 18 kDa of the different FPLC-subfractions of fraction II (Fig. 7) were submitted to "in gel" digestion and microsequencing studies. In the samples Ib, IIb and IIc the band at 13.0–14.0 kDa contained, as the only component, the bovine seminal fluid protein (aSFP). The 18 kDa band of IIa′ contained osteopontine (OPN) and HE4 proteins. The 18 kDa band of IIb contained BSP-A3 protein and the 18 kDa band of IIc contained the PDC-109 glycoproteins. The aSFP and PDC-109 (glyco)proteins had been previously identified by SDS-

Table 5 Molecular weights and its interpretation as corresponding to a family of a protein (12730 Da) and its glycoforms. Composition of the oligosaccharide chains.

M. w. of the glycoproteins	M. w. of the olygosaccharide chain		Composition of the oligosaccharide chains			
Da[x]	Exp.	Calc. Da[v]	N-acetyl glycosamine	i-hexose[z]	e-hexose[y]	Neuraminic acid
12730[w]						
13248	518	509	1			1(N-acetyl)
13405	675	671	1	1		1(N-acetyl)
13446	716	706	1	2	1	
13556	826	833	1	2		1(N-acetyl)

[z] Internal hexose, [y] external hexose, non-reducing end-chain hexose. [x] The molecular weights are affected by about 0.1–0.2% error inherent to the method but also by a minor shift of the m/z value owing to the partial superposition of the signals. [w] Peaks appearing in the different fractions. [v] The m.w. of the oligosaccharide chains is obtained as the difference between the m.w. of the supposed glycoproteins and that of the protein.

PAGE-sequencing analysis in fractions Ib and III (not shown in Fig. 1), respectively (Marquinez et al., 2000).

Major peaks in the UV-MALDI-MS spectra of fraction II (Fig. 6a and b) were obtained in the range 12.8–14.0 kDa (12735, 12907, 12985, 13465, 13520 and 13702 Da) together with minor ones at 6300–7050 Da and 10130 Da. On expansion of the scale (6–14 kDa, spectra not shown) peaks at 12735, 12800, 12907, 12940, 12985, 13160, 13356, 13465, 13520 and 13644 Da were detected. The major proteins are the bovine seminal fluid protein (aSFP) (12903 Da (Calvete et al., 1996)) together with BSP-A3 (12800 Da (Calvete et al., 1996)) and BSP-A1 (13644 Da (Calvete et al., 1996)). Most of the other molecular weights correspond to members of the enlarged BSP-family (Table 3) or of the new one (Table 4) while those peaks at 12940 Da and/or 12985 Da could be osteopontin (Cancel et al., 1999; Denhardt and Guo, 1993; Moura, 2005; Masuda et al., 2000) (see later, genetic polymorphysm). The neat peak at 10132 Da, which confirms that sporadically appearing in fraction1, was assigned to HE4, the human epididymis-specific protein found also in the bovine epididymus and in other species (Kirchhoff et al., 1991). HE4 is a putative secretory polypeptide of only 95 amino acids, the molecular mass of which was calculated to be approximately 10 kDa (Kirchhoff et al., 1991). A tentative recognition site for glycosylation (Asn-X-Thr) was located at position 14 of the polypeptide (Kirchhoff et al., 1991). It is worth of note the identification of osteopontin (OPN) in the Con A "non-interacting" fraction (fraction IIa) of the BSP (Marquinez et al., 2000), with an apparent molecular weight of 18 kDa by SDS-PAGE- sequencing analysis. Bovine OPN is a highly acidic (glyco)protein with a predicted m.w. of 41 kDa but isoforms of 14 and 55 kDa have been found in b.s.p. and accessory cell glands (Cancel et al., 1999; Denhardt and Guo, 1993; Moura, 2005; Masuda et al., 2000). Differences in molecular sizes of OPN from several origins have been attributed, in part, to the acidic nature of the protein which affect its mobility in SDS-PAGE (Iborra et al., 1996). The 55 kDa protein was glycosylated but carbohydrate was not detected in the 14 kDa fragment (Calvete et al., 1996; Denhardt and Guo, 1993), which was supposed to be a cleavage product of the 55 kDa OPN. The N-glycan structures of an OPN from human bone was of the biantennary complex-type (Moura, 2005), therefore if the 14 kDa fragment contain oligosaccharides with that structure it would have been retained by the lectin (Nachbar et al., 1976), which was not. Several peaks were found in the range 6300–7050 Da (Fig. 6 b). Some of them are double-charged ions of the (glyco)proteins in the 13000–14000 zone but the others (6385, 6457, 6510, 6568, 6620, 6687, 6755, 6781, 6842, and 6947 Da) could be assigned to caltrin (SDS-PAGE m.w. 6500 Da)(Clark et al., 1993) or any other positive modulatory factors present in the bovine seminal plasma (Clark et al., 1993) (see fraction 1).

CONCLUSIONS

In summary, the application of UV-MALDI MS technique to fractions of the bovine seminal plasma permits some conclusions:

a) The elimination of major amounts of low molecular weight (less than $\approx$ 10 kDa) material and/or the possibility of working with different sensitivities allow the production of well-defined spectra in the mass/charge range 5.5–20 kDa, with concentration of the most important peaks in the zone 12–14 kDa. Peaks in the range 20–90 kDa were small, possible due to the low concentration in the seminal fluid of the corresponding products, to the suppression effect of lower mass compounds and/or that they were remaining of complexes not totally disaggregated before the volatilization/ionization step. The appearance in the lower zone of the spectra (less than 10 kDa) of peaks that were not double-charged ions of the major (glyco)proteins indicates that protein fragments, peptides like caltrin and/or any other positive modulating factor of similar masses were complexed forming aggregates of high molecular weight.

b) The bovine acidic seminal fluid protein (aSFP) eluted with apparent molecular mass of 25

kDa from gel filtration chromatography and showed an apparent M_r 14 kDa by SDS-PAGE (Calvete et al., 1996). It is a homodimer which, until now, dissociated only in presence of SDS (Calvete et al., 1996). The molecular mass of the monomer calculated from the cDNA sequence of the aSFP was 12923 Da (Wempe et al., 1992), corresponding exactly to that obtained by UV-MALDI MS of the pure compound (Calvete et al., 1996) and very near to those obtained in this work (12925–12930 Da), in complex mixtures of (glyco)proteins. Three major (glyco)proteins (BSP-A1, BSP-A2 and BSP-A3) of the bovine seminal plasma showed by SDS-PAGE apparent molecular weights in the range 15.0–16.5 kDa (Manjunath and Sairam, 1987). Whereas BSP-A3 showed an apparent M_r 17–20 kDa by gel filtration at different pH values, the glycoproteins BSP-A1 and BSP-A2 were eluted as aggregates with M_r in the range 60–120 kDa at pH 7 or above. However, at acid pH_s or in 8M-urea-buffer they showed M_r 17–20 kDa (Manjunath and Sairam, 1987). These M_r are much higher than those calculated by structural analysis of the BSP-A3 protein (12774 Da) (Seidah et al., 1987) or of the PDC–109 mixture of both glycoproteins (BSP-A1 plus BSP-A2) 13443 (Seidah et al., 1987; Salois et al., 1999). On the other hand, these calculated values are in very good agreement with those determined in fractions isolated of the seminal fluid (BSP-A3: 12789 Da and PDC-109: 13444 Da) (Calvete et al., 1996) and with those obtained in the present work [BSP-A3: 12801–12810 Da, Bsp-A2: 13356 Da, BSP-A1: 13465 Da and PDC-109: 13343 Da]. The above examples clearly demonstrated that the UV-MALDI-determined molecular weights are those of the monomer units and that they can be obtained without previous extensive fractionation and/or purification of the sample.

c) The only previously reported glycoproteins in bovine seminal plasma were those of the BSP-family (BSP-A1 and BSP-A2) (Manjunath and Sairam, 1987). In this family the difference of molecular weight between the protein and the major glycosylated form (BSP-A1) was 655 Da, corresponding to the only carbohydrate chain of the glycoprotein, with a NeuAc (α2→3) Gal (β1 →3) GalNAc structure (Calvete et al., 1994; Gerwig et al., 1995). The MALDI spectra of fractions of bovine seminal plasma showed peaks of the mother protein (BSP-A3), major (BSP-A1) and minor (BSP-A2) glycoforms and others which difference in their molecular weights with that of the BSP-A3 could be expressed in terms of monosaccharide composition of the unique carbohydrate chain (Table 3). This suggested that the BSP-family is more extended than previously supposed, differing its members in the size and composition of the oligosaccharide chain (Table 3). The UV-MALDI spectra of fractions 2, 3 and subfraction II showed new families starting from proteins with molecular weights about 12730 Da and 12756 Da respectively, which glycoforms have also only one oligosacharide chain of the same type and composition of the BSP-family (Tables 4 and 5). The low carbohydrate content of the bovine seminal plasma glycoproteins and the simple composition of its oligosaccharide chains contrast with those of the mucin glycoproteins of human seminal plasma (Hanisch et al., 1985; Hanisch et al., 1986).

d) The amino acid sequences of BSP-30-kDa and BSP-A3 proteins obtained from two different bulls differ in one (BSP-30-kDa) and two amino acids (BSP-A3), namely: proline instead of serine in the first case and glutamic acid/isoleucine which were replaced by glutamine and lysine in the second (Seidah et al., 1987). These differences were supposed to be due to polymorphism in the genes coding for the BSP proteins (Seidah et al., 1987). It is worth of note that the mother proteins of the three families differ only in about 60–70 Da in the first case and 30–40 Da in the second. Sets of peaks differing in a few Da (i.e., spectrum Fig. 5c: [12800, 12835, 12850, 12875], [12993, 13003, 13038], [13146, 13152], [13248, 13261], etc) appear in fraction 3, possible due to the excelence of the 3b spectrum not obtained in other cases. These differences are in the range of the molecular mass of one amino acid unit or of the difference between replaced amino acids and are consistent with the before mentioned genetic polymorphism, considering that the seminal plasma used in this work was obtained from a pool of semen samples.

e) The fact that all the fractions have similar compositions indicates that the fractionation of

the BSP was not due to specific affinity interactions between the Con A lectin and the carbohydrate chains of the glycoproteins. This was not detected in a previous fractionation (Marquinez et al., 2000) as only the "non-interacting" fraction was studied. Considering the highly hydrophobic character of the proteins of BSP and the existence of a hydrophobic site in the Con A lectin (Goldstein and Hayes, 1978; Edelman and Wang, 1978), it is probable that the separation was due to non-specific hydrophobic interactions. Secondary hydrophobic effects in a Con A affinity chromatography have been detected previously (Nachbar et al., 1976). This type of interaction is consistent with the bleeding shapes of the elution curves (Marquinez et al., 2000) and with the methyl gluco and mannopyranosides eluents acting as polarity-reducing compounds (Shaltiel, 1977). The major components in the fractions (fractions 2 and 3) retained by the column were Con A affinity non-interacting products indicating the absence of major amounts, in the bovine seminal plasma, of biantenary complex-type, hybrid-type and high mannose-type oligosaccharide-containing glycoproteins (Yamamoto et al., 1993).

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